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(71) Applicant: **Max-Planck-Gesellschaft zur Förderung der Wissenschaften e.V.**
Bunsenstrasse 10
D-3400 Göttingen(DE)

(72) Inventor: **Leemans, Jan, Dr. c/o Plants Genetic Systems n.v.**
J. Plateaustraat 22
B-9000 Gent(BE)
Inventor: **Deblaere, Rolf, Dr. c/o Plants Genetic Systems n.v.**
J. Plateaustraat 22
B-9000 Gent(BE)

(74) Representative: **Vosslus & Partner**
Siebertstrasse 4 P.O. Box 86 07 67
D-8000 München 86(DE)

(54) **Stable binary agrobacterium vectors and their use.**

(57) Binary vectors for the introduction of DNA into plant cells are presented. They contain the origin of replication of the Pseudomonas plasmid pVS1 and a T-DNA region consisting of the border sequences of the T_L-DNA of the pTiB6S3 plasmid and a nos-NPTII chimaeric gene to select kanamycin resistance in plant cells. The vectors replicate completely stably in Agrobacterium. Transformation of tobacco protoplasts using the binary vector is equally efficient as for previously described vector systems. The transferred T-DNA is delineated by the border sequences and in general only one or few copies of the T-DNA become integrated into the plant genome.

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"Stable Binary Agrobacterium Vectors and their Use"

Transfer of foreign DNA into plant genomes is most often performed using plasmid vector systems derived from the tumor-inducing plasmids of Agrobacterium tumefaciens. T-DNA, a fragment of these plasmids, is transferred into the plant chromosomes during the naturally occurring process of crown gall tumor formation (for a review see Gheysen et al, 1985). The host range of Agrobacterium is very wide and recently, transformation of Liliaceae by Agrobacterium has been reported (Hernalsteens et al, 1984). This contradicted the classical observations, which indicated that the applicability of the Agrobacterium system would be limited to dicotyledonous plants. However, Agrobacterium -mediated transformation of Gramineae, including the important cereal crops, has not yet been achieved. Other emerging techniques to introduce foreign genes into plants are the uptake of naked DNA by protoplasts after polyethylene glycol treatment, electroporation or a combination of both. Although these methods can be applied to both monocot and dicot species, the use of protoplasts very often limits the possibilities to regenerate normal plants after transformation.

At present, a wide variety of Agrobacterium vector systems has been developed. These allow easy cloning of foreign genes and they are transferred in a single step from E. coli into A. tumefaciens. Most important, transformation can be obtained after infection of wounded plant parts, such as stems, leaf segments, tuber slices or root slices. Two observations allowed the design of the presently used Agrobacterium vectors: first the T-DNA genes responsible for phytohormone -independence of crown gall cells are not required for T-DNA transfer and integration. Second, the T-DNA borders, including the directly repeated sequences of 25 bp were shown to be the only essential cis-acting sequences involved in T-DNA transfer.

One type of Ti-plasmid derived vectors are intermediate or shuttle vectors which recombine through homologous recombination with a receptor Ti-plasmid (Zambryski et al, 1983). A second type are binary vector systems (Bevan et al, 1984) in which T-DNA is carried by a vector molecule, capable of autonomous replication in Agrobacterium. Ti-plasmid functions required for T-DNA transfer are complemented in trans by the vir genes, present on a Ti-plasmid derivative (Hoekema et al, 1983). Agrobacterium strains are equally easy to construct using recombination or binary vectors, but the use of binary vectors omits the need to verify the structure of the recombinant Ti-plasmid by Southern blot and hybridization analysis. Most important, it is hoped that complete libraries of plant genomic DNA, constructed in cosmid derivatives of binary vectors, can be transferred 'en masse' into plant cells. This could allow the complementation of biochemical mutants or selection for the expression of dominant genes. In the resulting plant cell lines the gene of interest would be easily recognized due to the cotransferred T-DNA sequences. This would enable the cloning of this gene. Such experiment would involve a large number of clones and the stability of the binary vectors will determine whether complete libraries can be successfully transferred. The binary vectors presently described are based on broad host range plasmids, such as pRK252 (Bevan, 1984) and pTJS75 (An et al., 1985), which are derived from the P-type plasmid RK2. However, a major consideration in the use of these replicons is their considerable instability under non-selective conditions (Ditta et al., 1985). Therefore there existed a need for other plasmids which might lead to more stable Agrobacterium replicons.

It is the object of the present invention to provide novel binary vectors for the introduction of foreign DNA into plant cells which vectors allow easy cloning, are mobilizable at high frequency to Agrobacterium where they replicate autonomously and which display improved stability under non-selective conditions.

This object is achieved on the basis of the surprising finding, that binary vectors containing the origin of replication of the Pseudomonas plasmid pVS1 show the above mentioned desirable characteristics.

pVS1 is a 30kb Pseudomonas aeruginosa plasmid that confers resistance to mercury ions and sulfonamide (Stanisich et al., 1977). pVS1 can be efficiently mobilized into Agrobacterium by the P plasmid RP1 (Itoh et al., 1984).

The subject matter of the invention are novel binary vectors for the introduction of foreign DNA into plant cells which comprise, the origin of replication of the Pseudomonas plasmid pVS1, an origin for replication in E. coli, and a T-DNA region of the T₁-DNA of the octopine-type Ti-plasmid provided with different adapter fragments, transcription regulatory signals and flanked by the right and left border sequence.

In accordance with the present invention the construction of a novel binary vector system for Agrobacterium-mediated transformation of plant cells is achieved. Binary vectors provide a practical advantage over "classical" vector systems in that they do not require to integrate the vector in a resident Ti-plasmid through homologous recombination.

The pVS1-derived vectors of the invention have following useful properties:

- They are sufficiently small to allow easy cloning (ca 10 kb).
- They are mobilizable at high frequency to Agrobacterium.
- The origin of replication (4 kb fragment) is derived from the 30kb pVS1 plasmid and hence is able to replicate recombinant plasmids of relatively large size.
- T-DNA transfer using the binary vectors of the invention is equally efficient as with recombinational vectors. Hence, trans complementation of the vir-gene products appears fully effective.
- If binary vectors are to be used to perform shotgun cloning experiments, the pVS1 derived vectors will prove very useful since they do not exhibit any instability, neither under normal growth conditions, nor during plant transformation.

The binary vectors of the present invention are a useful tool for the construction of gene libraries, i.e. of genomic libraries or of cDNA libraries. Preferred host organisms are Agrobacterium.

From the molecular analysis of the T-DNA structure in 6 transformed cell lines it is clear that:

- 1) T-DNA borders are recognized to delineate most of the T-DNA inserts.
 - 2) The copy number of the inserts is not different from those obtained using recombinational vectors.
- The invention will now be described in more detail with reference to the accompanying drawing.

Figure 1. Construction of the pVS1 derived binary vector pGV941.

A 3.8kb BamHI/SacII fragment of pVS1, carrying the stability and replication functions was cloned into the BamHI/Sall site of pGV867, producing pGV922. SacII and Sall sticky ends were converted to blunt end by DNA polymerase treatment prior to cloning. The unique BamHI site of pGV922 was eliminated by filling in the sticky ends and religating the plasmid, resulting in pGV940. A 1.87kb EcoRI/HindIII fragment, isolated from pGV815 (Deblaere et al., 1985) and containing the octopine T_L-DNA border sequences, was cloned into the EcoRI/HindIII sites of pGV940, producing pGV943. The chimaeric NPTII gene from mGV2 was isolated as a 2.4kb BamHI fragment and inserted into the unique BglII site of pGV943. The resulting plasmid pGV941 contains a unique BamHI and HpaI site for cloning. This construct was deposited with DSM Gottingen, F.R.G. and received the number

Abbreviations: Cb: carbenicillin resistance; Cm: chloramphenicol resistance; Neo: neomycin resistance for plants; Sm: streptomycin resistance; B: BamHI; Bg: BglII; E: EcoRI; h: HindIII; Hp: HpaI; P: PstI; S: Sall; rep: replication function; sta: stability function.

Figure 2. Physical analysis of the T-DNA structure in pGV944 transformed cell lines.

A. The restriction map of the T-region of pGV944. The dotted areas represent the vector part of pGV944. The wavy lines indicate the T_L-DNA border sequences. The fragments used as probe are indicated.

B. Hybridization pattern of 6 independent cell lines transformed with C58C1Rif^R(pGV2260)(pGV944). 10 ug of total plant DNA was digested with BclI, separated by agarose gel electrophoresis, blotted onto nylon filter and hybridized to radioactively labelled probes :

Probe 1: the 1.0 kb BclI/Sma fragment from pKC7 (Rao and Rogers, 1979) covering the Km^R structural gene;

Probe 2: the 1.4 kb BamHI/PvuII fragment, containing the ocs structural gene and isolated from mGV1 (Deblaere et al., 1985);

Probe V: the plasmid pGV922.

C Representation of a tandem duplication configuration of the pGV944 T-DNA; the size of the expected BclI fragments is indicated. Bands corresponding to a possible junction fragment are indicated in B by an asterisk.

Materials and methods

Bacterial strains and plasmids.

5 E. coli strain K514 (Colson et al, 1965) was used throughout the recombinant DNA work. Agrobacterium strain C58C1Rif^R (pGV2260) was used to provide in trans the vir-functions for the binary vectors (Deblaere et al, 1985).

pGV867 is a pBR325 derivative carrying a 2.25kb HindIII/BamHI fragment which encodes streptomycin and spectinomycin resistance. pGV815 and pGV825 are intermediate vectors, containing an avirulent T-DNA
10 (Deblaere et al, 1985).

pGV2486 is a cointegrate between pGV2260 and pGV891. pGV891 is similar to pGV833 (Deblaere et al., 1985): the chimaeric NPTII gene was isolated as a BamHI fragment from mGV2 - derived from M13mp7 with a Pnos-neo-3'-ocs cassette - and inserted in the unique BglII site of pGV825. The ocs gene was subsequently inserted as a BamHI fragment as previously described for the construction of pGV833.

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DNA techniques

Recombinant DNA techniques were performed as described (Maniatis et al., 1982).

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Bacterial conjugations.

Binary vectors were mobilized from E. coli to A. tumefaciens in a triparental mating using helper
25 plasmid pRK2013 (Ditta et al, 1980). 0.1 ml of exponentially growing cultures of donor, acceptor and helper strain were plated on a LB agar plate, and incubated overnight at 28°C. The mobilization mixture was recovered in 2 ml 10⁻² M MgSO₄ and serial dilutions were plated on selective medium. Transconjugants were selected on minimal medium containing streptomycin (1000 ug/ml) and spectinomycin (300 ug/ml). The skilled practitioner knows other strains of E. coli and of Agrobacterium which provide in trans the vir
30 functions as well as other helper plasmids which can equally be used for carrying out the present invention.

Plant transformations.

35 Agrobacterium strains carrying the binary vector were tested in a protoplast cocultivation system. Mesophyll protoplasts were isolated from Nicotiana tabacum cv. Petit Havana SR1 (Maliga et al, 1973) and cocultivated as described by Marton et al. (1979). Transformed plant cells were selected for kanamycin resistance as described by De Block et al. (1984). Octopine synthase activity in transformed cell lines was assayed according to Lœmans et al (1981).

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T-DNA analysis.

Total plant DNA was isolated as described previously (Dellaporta et al, 1984). Digested plant DNA was
45 transferred to a Hybond N nylon membrane (Amersham), using the classical Southern blot procedure. DNA-DNA hybridization was performed according to the suppliers instructions.

Example

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A) Construction of binary vectors.

The stability and replication functions of the Pseudomonas plasmid pVS1 are carried on a 3.8kb BamHI/SacII restriction fragment (Itoh et al., 1984). This fragment was used in the construction of plasmid
55 pGV943 (see figure 1). This binary vector contains a segment of pBR325, providing carbenicillin resistance and replication functions. The latter are necessary since pVS1 does not replicate in E. coli. pGV943 carries

a bacterial streptomycin-spectinomycin resistance marker to facilitate selection in *Agrobacterium*. A 1.87kb T-DNA fragment, recloned from the cointegration vector pGV815 (Deblaere et al., 1985) and containing the left and right border sequences of the octopine T₂-DNA, was included. This avirulent T-DNA contains unique restriction sites for BamHI, BglII and HpaI cloning.

To be used as a control, the binary vector pGV912 was constructed, based on the broad host range replicon pRK404 (Ditta et al., 1985) and containing an identical T-DNA as pGV943. A 2.8kb PvuII fragment, carrying an avirulent T-DNA and the streptomycin-spectinomycin resistance gene, was isolated from pGV825 (Deblaere et al., 1985) and cloned into the SmaI site of pRK404, producing pGV912. The stability and plant transforming properties of pGV912 are compared to those of pGV943.

To successfully apply these binary vectors in plant transformation experiments, a chimaeric neomycin phosphotransferase II (NPTII) gene was included as dominant selectable marker gene (Herrera-Estrella et al., 1983; Bevan et al., 1983; Fraley et al. 1983). The chimaeric NPTII gene was isolated from pLGVneo1103 (Lörz et al., 1985) as a 2.3kb EcoRI/SalI fragment. It was converted to blunt-end by treatment with the Klenow fragment of the *E. coli* DNA polymerase and subcloned in the HincII site of M13mp7 (Vieira and Messing, 1982), producing mGV2. The NPTII gene was recloned as a BamHI fragment and inserted in the unique BglII site of either pGV912 and pGV943, resulting in pGV915 and pGV941 respectively. The construct pGV941 was deposited with the Deutsche Sammlung für Mikroorganismen (DSM) and received the deposition no. 3893.

In a model experiment, the octopine synthase (*ocs*) gene was isolated from mGV1 as a 1.4kb BamHI fragment and inserted in the unique BamHI site of pGV915 and pGV941 as described previously for the cointegration vector (Deblaere et al., 1985), producing pGV939 and pGV944 respectively. The *ocs* gene was chosen to detect gene expression of additionally introduced genes in the T-DNA, because *ocs* activity can easily be detected in transformed plant cells (Leemans et al., 1981).

B) Stability of binary vectors in *Agrobacterium*.

The binary vectors pGV939 and pGV944 were mobilized from *E. coli* to *Agrobacterium* strain C58C1Rif^R(pGV2260) in a triparental mating using HB101(pRK2013) as a helper. Streptomycin-spectinomycin resistant transconjugants arose at a frequency of 1% in both crosses. The stability of the vector plasmids was determined in cultures of C58C1Rif^R(pGV2260)(pGV939) and C58C1Rif^R(pGV2260)(pGV944), grown overnight in selective LB medium, containing streptomycin (300ug/ml) and spectinomycin (100ug/ml). Bacteria were plated on non-selective LB agar medium and resulting colonies were screened for spectinomycin resistance. Furthermore, the cultures were diluted and grown for 7, 14 and 21 generations in non-selective liquid medium before plating on non-selective LB plates. Again, colonies were screened for their spectinomycin resistance. Table 1 shows that pVS1 and its derived plasmids are completely stable in *Agrobacterium*, whereas pRK404 and its derivative pGV939 are lost at high frequency during non-selective growth.

Table 1

Plasmid stability in *Agrobacterium* strain C58C1Rif^R

Number of generations	pVS1	pGV922	Plasmids pGV944	pGV939	pRK404
0	>99	>99	>99	76	68
7	>99	>99	>99	74	60
14	>99	>99	>99	56	54
21	>99	>99	>99	44	45

The numbers indicate the percentage of antibiotic resistant bacteria after screening of the non-selectively grown cultures. pVS1 was screened for sulfathiazole resistance (250ug/ml), pRK404 for tetracycline resistance (5ug/ml) and the other plasmids for spectinomycin resistance (100ug/ml).

The loss of both vectors was also measured under conditions of a classical tobacco protoplast cocultivation experiment. 10ul of a selectively grown bacterial culture was added per 1ml of protoplast suspension. Every 24 hours a sample was taken from the medium and the bacteria were screened for antibiotic resistance. Again the pVS1 derived pGV944 proved completely stable (>99% spectinomycin resistance), whereas 24 hours after infection, only 68% of the Agrobacterium cells still contained pGV939.

C) Plant cell transformations.

Mesophyll protoplasts were isolated from Nicotiana tabacum cv Petit Havana SR1 and cocultivated with the following strains: C58C1Rif^R(pGV2260)(pGV939), C58C1Rif^R(pGV2260)(pGV944) and C58C1Rif^R(pGV2486). pGV2486 contains an identical T-DNA as the binary vectors, but in a cointegration vector. Transformed plant cells were selected for kanamycin resistance (50ug/ml) on solid medium as described by De Block et al.(1984). Kanamycin resistant calli were obtained at a frequency of 4% for the cointegration vector pGV2486 and 1 and 5% for the binary vectors pGV939 and pGV944 respectively.

Kanamycin resistant calli were further cultured on solid medium containing 50ug/ml kanamycin and screened for octopine synthase activity. A 100% correlation between kanamycin resistance and octopine synthesis in clones transformed with C58C1Rif^R(pGV2260)(pGV944) was observed. In 24 clones transformed with the pRK404 derived pGV939, as well as with pGV2486, 17 showed variable levels of octopine synthase activity and in 7 calli no octopine production was detected.

D) T-DNA structure in transformed cell lines.

Total plant DNA from 6 kanamycin resistant pGV944 transformed cell lines was analysed by Southern blotting to determine T-DNA copy number and structure. Hybridization to probes derived from the left and right part of the T-DNA allows one to accurately determine the T-DNA copy number. The hybridization pattern reveals that lines 3, 4 and 6 have only a single T-DNA insert, while multiple inserts are present in lines 1 (three copies), 2 (two copies) and 5 (> four copies) (see figure 2).

To investigate whether the T-DNA border sequences have been recognized to delineate the inserted T-DNA, pGV922 was used as probe. It contains all vector sequences present in pGV944, but lacks the T-DNA. Only one of the T-DNA copies in cell line 5 hybridized to vector sequences.

Cell line 1 contains a 5 kb fragment, which hybridizes to both the left and right side of the T-DNA. This is characteristic for a tandem organization of the multiple T-DNA inserts. A similar junction fragment is also observed in line 5, but not in line 2.

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Claims

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1. A binary vector for the introduction of foreign DNA into plant cells which comprises the origin of replication of the Pseudomonas plasmid pSV1 and a T-DNA region comprising the border sequences of the T_L-DNA of an octopine Ti-plasmid.

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2. The vector of claim 1 which additionally contains a selectable marker for plant transformation, transcription regulatory signals and adaptor fragments in between the border sequences and optionally bacterial selection markers.

3. The vector of claim 2 wherein the selectable marker for plant transformation is a chimeric kanamycin resistance gene.

4. The vector of any of claims 1 to 3 which is pGV941 (DSM No. 3893).

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5. The vector of any claims 1 to 4 which additionally contains a gene encoding a gene product of interest.

6. The vector of claim 5 which is pGV944.

7. A gene library which is stably replicable in Agrobacterium comprising a binary vector of any claims 1 to 4.

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8. A host cell transformed with a binary vector of any of claims 1 to 6.

9. The use of the origin of replication of the Pseudomonas plasmid pVS1 for the construction of a binary vector which is stably replicable in Agrobacterium.

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FIGURE 1

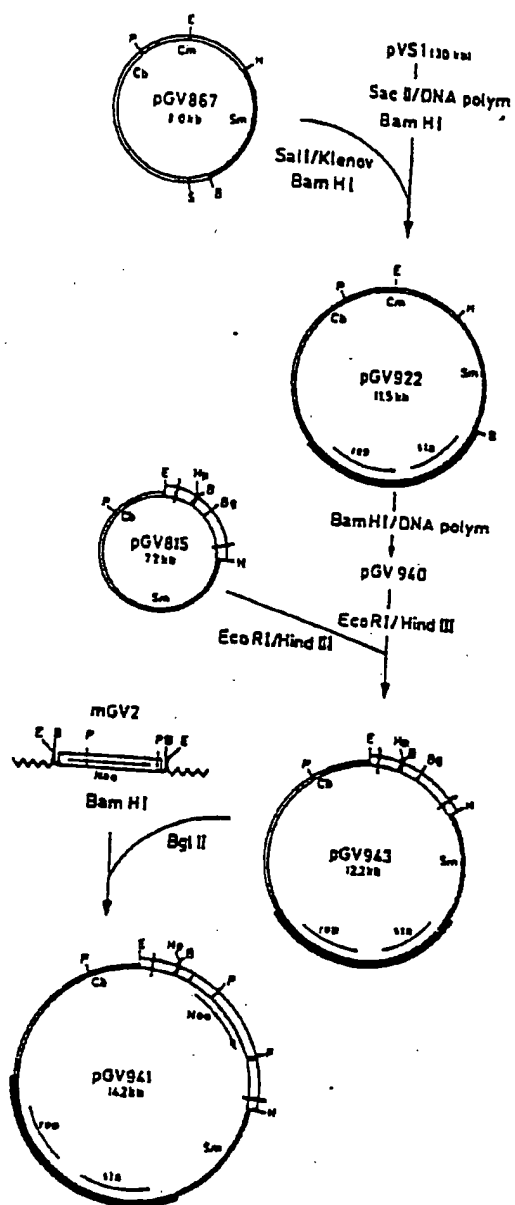
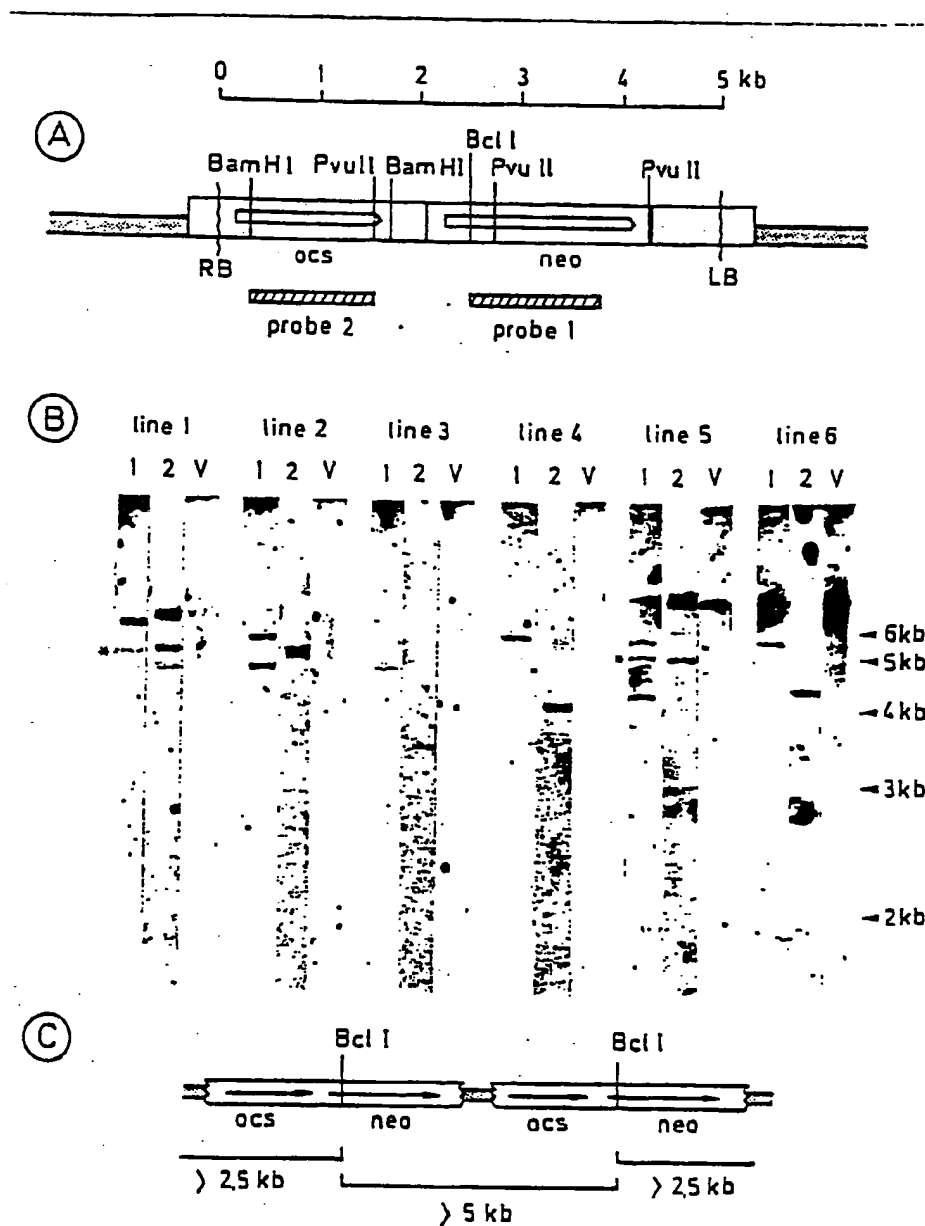


FIGURE 2





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The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 20-07-1987	Examiner MADDOX A.D.
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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A	--- WO-A-8 504 899 (AGRACETUS) * Claims 9-11 *	1-9	
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<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>			

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The present search report has been drawn up for all claims					
Place of search THE HAGUE		Date of completion of the search 20-07-1987	Examiner MADDOX A.D.		
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document</p>					

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DOCUMENTS CONSIDERED TO BE RELEVANT			Page 4
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
D,A	NUCLEIC ACIDS RESEARCH, vol. 13, no. 13, July 1985, pages 4777-4788, IRL Press Ltd, Eynsham, Oxford, GB; R. DEBLAERE et al.: "Efficient octopine Ti plasmid-derived vectors for agrobacterium-mediated gene transfer to plants" * Whole document *	1-9	
E	EP-A-O 223 417 (LUBRIZOL) * Claims 1-3 *	1-3,8	
			TECHNICAL FIELDS SEARCHED (Int. Cl. 4)
The present search report has been drawn up for all claims			
Place of search THE HAGUE		Date of completion of the search 20-07-1987	Examiner MADDOX A.D.
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